



SUB D1)
B1
cut

Artificial Skin, a brand of artificial skin, is sold by Integra LifeScience Corporation of Plainsboro, New Jersey, USA, and has been approved by FDA for use in the USA since 1996. INTEGRA™ Artificial Skin is a bilayer biosynthetic sheet comprising porous collagen-glycoaminoglycan integrated with a thin silicone membrane as an outer layer. The use of such a porous collagen-glycoaminoglycan artificial skin as a biocompatible acellular dermal replacement in deep and full-thickness burn wounds is well known.

B. Please amend the paragraph bridging pages 1 and 2 to recite:

SUB D2)
B2

It has been observed that within about 14 to 21 days following the grafting of a bilayer biosynthetic sheet comprising porous collagen-glycoaminoglycan integrated with a thin silicone membrane as an outer layer, there is full vascularization of the neodermis formed in the artificial skin. Thereafter an ultra thin split thickness skin graft must be harvested from a donor site in order to cover the neodermis immediately after the silicone membrane is removed. Substantial research effort has been undertaken in the past to determine the possibility of reliably grafting CEA on the neodermis, since an effective combination of CEA and such a dermal replacement as a biosynthetic collagen-glycoaminoglycan dermal matrix should eliminate the second operative stage, the associated pain and scarring, as well as a need for a second donor site, which may not be available in extensively burned patients. If the grafted CEA does not 'take' on the neodermis after the silicone membrane is peeled off, it can be replaced by another CEA. Whereas, in the conventional application of a such a biosynthetic collagen-glycoaminoglycan dermal matrix dermal replacement, another split thickness autograft must be harvested from a second or even a third donor site. There have been very limited initial anecdotal reports on experience with such a combination technique, such as Sheridan *et al.* 1999 and Pandya *et al.* 1998. At the 10th Congress of International Society for Burn Injuries, November 1998 in Israel, the difficulties with the conventionally cultured graft anchoring onto such a biosynthetic collagen-glycoaminoglycan neodermis were addressed. The exact reasons for such difficulties remain unknown.

C. Please amend the first full paragraph on page 2 to recite:

SUB D3)
D3

LASERSKIN™ artificial skin is another artificial skin material. This material is made of a thin (e.g., about 20 μ m-thick membrane) and pliable biosynthetic membrane comprising a 100% benzyl esterified hyaluronic acid derivative suitable for use as a

SUB D3
B3
cont

substratum in the growth of skin cells. The membrane is drilled by a laser to have a series of holes or microholes typically of about 40 μ m in diameter to allow the ingrowth and proliferation of keratinocytes. In addition, a series of larger holes (about 0.5 mm in diameter) are also typically provided to allow drainage of wound fluids. LASERSKINTM artificial skin is available from Fida Advanced Biopolymers Ltd. (Abano Terme, Italy). The recommendation of the LASERSKINTM artificial skin manufacturer is to seed human keratinocytes on the LASERSKINTM artificial skin material preseeded with irradiated 3T3 cells as feeder layer. When seeding human keratinocytes on LASERSKINTM artificial skin preseeded with irradiated 3T3 cells as feeder layer, it was found that, after the initiation of the formation of keratinocyte colonies, the xenogenic 3T3 cells growing on the material were less likely to be washed away than those growing on a culture dish as in the conventional Green's method during each flushing procedure with phosphate-buffered saline (Rheinwald and Green, Cell, 6:331-344 (1975)). It is believed that the remaining 3T3 cells or debris might have sensitized the host to xenogenic antigen resulting in undesired late graft rejection. What is needed is a cultivation and engraftment procedure with a biocompatible, durable human skin substitute.

D. Please replace the first paragraph of the Summary on page 4 with the following recital:

SUB D4
B4

According to the invention, autologous cultured keratinocytes grown on a biocompatible substratum are engrafted on the neodermis of artificial skin covering a wound. In one of its aspects, the invention provides a method for cultivation of graftable skin by growing a layer of dermal fibroblasts upon at least an upper side of a biosynthetic substratum of a derivative of benzyl esterified hyaluronic acid; and, after the dermal fibroblast layer grows to become at least sub-confluent, growing a layer of keratinocytes from cells harvested from the intended recipient/target donor patient over these dermal fibroblasts to form a composite skin graft material.

In another of its aspects, the invention provides a method for the cultivation of a graftable skin by growing a layer of keratinocytes from cells harvested from the intended recipient/target donor patient upon an upper side of a biosynthetic substratum of a derivative of benzyl esterified hyaluronic acid to form a composite skin graft material.

In another of its aspects, the invention provides a graftable skin material comprising a composite of a biosynthetic substratum of a derivative of benzyl esterified hyaluronic acid; a

SUBP
cont

layer of dermal fibroblasts upon at least an upper side of the biosynthetic substratum; and a layer of keratinocytes grown from cells harvested from the intended recipient over the dermal fibroblasts upon the upper side of the substratum.

BY
cont

In another of its aspects, the invention provides a graftable skin material comprising a composite of: a biosynthetic substratum of a derivative of benzyl esterified hyaluronic acid; a first layer of dermal fibroblasts upon a first basal side of the biosynthetic substratum; a second layer of dermal fibroblasts upon a second upper side of the biosynthetic substratum; and a layer of keratinocytes grown from cells harvested from the intended recipient over the dermal fibroblasts grown upon the upper side of the substratum.

In another aspect, the invention provides a method for grafting a graftable skin material by applying an artificial skin substrate upon a wound bed of a recipient patient; the artificial skin substrate comprising a layer of collagen-glycoaminoglycan on a basal side to be juxtaposed to said wound bed and a covering membrane of silicone on an opposing upper side; allowing a sufficient time to form a vascularized wound bed under the collagen-glycoaminoglycan; thereupon removing the silicone membrane; and thereupon applying a basal side of a sheet of cultivated skin material over said collagen-glycoaminoglycan, said cultivated skin material comprising at least a layer of keratinocytes upon an upper side of a substratum, said keratinocytes being harvested from a target donor patient. In one embodiment, the cultivated skin material further comprises a layer of dermal fibroblasts upon at least an upper side of a biosynthetic substratum in which the layer of keratinocytes is over the dermal fibroblasts. In another embodiment, another layer of dermal fibroblasts is located upon the basal side of the biosynthetic substratum.

In each of the above aspects, the dermal fibroblasts may be allogenic or autologous to the keratinocytes.

Autologous keratinocytes may be cultivated on a biosynthetic membrane following pre-seeding with autologous or allogenic dermal fibroblasts. One such biosynthetic membrane comprises a 100% benzyl esterified hyaluronic acid derivative having a series of laser drilled holes or microholes typically of about 40 μ m in diameter to allow the ingrowth and proliferation of keratinocytes and a series of larger holes (about 0.5 mm in diameter) to allow drainage of wound fluids (LASERSKINTM artificial skin commercially available from Fidia Advanced Biopolymers Ltd., Abano Terme (PD), Italy). The resultant composite material may then be applied on the neodermis of artificial skin which had been previously

BY
Out

engrafted on the patient. The composite material, and specifically Composite Biocompatible Skin Graft (CBSG) material comprises autologous keratinocytes and allogenic or autologous dermal fibroblasts grown on the substratum. A method for fabricating the composite material includes the application of dermal fibroblasts onto the substratum as a feeder layer and then inoculating autologous keratinocytes on the resultant structure. A method for engraftment comprises first applying an artificial skin with a protective silicone membrane on a wound area, thereby allowing vascularization; following vascularization, removing the silicone membrane and engrafting the cultured composite material onto the vascularized artificial skin.

E. Please amend the paragraph bridging pages 4 and 5 to recite:

BS

Human fibroblasts used in the cultivation technique according to the invention were found to achieve a role similar to 3T3 cells in the initiation of keratinocyte colonies on LASERSKIN™ artificial skin. Specifically, it was found that the seeding efficacy of human keratinocytes was increased to up to 95%. CBSG containing autologous keratinocytes and autologous dermal fibroblast or allogenic dermal fibroblasts or a combination of autologous and allogenic dermal fibroblasts according to the invention, has been successfully applied to burn patients whose wounds were previously grafted with allografts.

F. Please amend the second full paragraph on page 5 to recite:

SUB. DS)
De

CBSG material according to the invention offers notable advantages. First, the basal proteins (including the early basement membrane proteins such as collagen IV and fibronectin) of the cultured graft are protected from dispase treatment because the keratinocytes are directly cultivated on a pliable laser drilled membrane. This is believed to enhance anchorage of the cultured keratinocytes on the neodermis. Second, in addition to acting as a feeder layer, the dermal fibroblasts in the inventive CBSG material evidently produce a number of proteins such as native collagen fibers and fibronectin which is believed to facilitate the attachment of a cultured graft. Third, the cultured keratinocytes of the inventive CBSG can be grafted five to seven days sooner than can traditionally-cultured keratinocytes. This is because cultured keratinocytes of the inventive CBSG are capable of being transferred and grafted at the sub-confluent or less differentiated stage rather than at a later confluent stage. Fourth, since there is minimal need for a donor site there is less likelihood of widespread scarring related to donor site harvesting. Fifth, the cultured

SUBDS cont B6 cont
keratinocytes of CBSG can be handled much more easily than the conventional CEA during its application on the neodermis of the artificial skin. Fewer cultured cells are lost or damaged during the transfer and application of CBSG. This should improve the success rate of the cultured graft. Sixth, the inventive engraftment technique can result in higher demand and broader scope of clinical applications for artificial skin.

G. Please amend the heading and paragraph between lines 11 and 19 on page 7 to recite:

Biosynthetic biocompatible substratum

SUB D6 B7
In the following experiments, LASERSKINTM artificial skin was used. LASERSKINTM artificial skin is a biosynthetic biocompatible substratum for keratinocyte cultivation according to the invention. LASERSKINTM artificial skin is a form of thin and pliable biosynthetic membrane comprising a 100% benzyl esterified hyaluronic acid derivative. This material is drilled with holes to facilitate the culturing of the keratinocytes thereupon. LASERSKINTM artificial skin is commercially available from Fidia Advanced Biopolymers Ltd., Abano Terme (PD), Italy. LASERSKINTM artificial skin was found to be useful to the inventors' experiments, although its preparation and application are not done according to the manufacturer's conventional instructions. There is nothing to preclude the use of compatible bioequivalents or human skin substitutes would also work in a similar fashion with the inventive CBSG.

H. Please amend the heading at the bottom of page 7 to recite:

B8
Comparison of seeding efficacy of human keratinocytes on LASERSKINTM artificial skin

I. Please amend all the paragraphs on page 8 to recite:

B9
Human dermal fibroblasts and 3T3 cells were grown separately on LASERSKINTM artificial skin with DMEM supplemented with 10% FBS. At sub-confluency, the 3T3 cells were treated with mitomycin-C (4mg/ml) for 2 hours at 37 °C, whereas the human fibroblasts were not treated. Human keratinocytes were seeded (3×10^4 cells/cm²) on top of the LASERSKINTM artificial skin either pre-seeded with 3T3 cells or human fibroblasts. The keratinocyte suspension was instilled and concentrated on the Laserskin surface (0.5ml/cm²).

Thirty minutes after seeding, DMEM supplemented with 10% FBS, insulin (4mg/ml), cholera toxin (6ng/ml), and EGF (10ng/ml) (per Rheinwald *et al.* 75) were added to the culture.

In a control run, human keratinocytes were also seeded on top of plain LASERSKIN™ artificial skin (Figure 1C). Efficacy was exhibited as hereinafter noted. After incubation at 37°C in a humidified atmosphere with 5% CO₂ for 24 hours, the non-seeded keratinocytes on the LASERSKIN™ artificial skin were gently rinsed off with PBS. The cells were then counted with a hemocytometer. In order to quantify the number of fibroblasts or 3T3 cells detached together with the non-seeded keratinocytes during the washing of the LASERSKIN™ artificial skin, the LASERSKIN™ artificial skin without keratinocytes but seeded with equivalent number of 3T3 cells or fibroblasts were included as control.

Comparison of seeding efficacy of rat keratinocytes on LASERSKIN™ artificial skin

The seeding of rat keratinocytes on LASERSKIN™ artificial skin was performed according to a technique essentially the same as the seeding of human keratinocytes. Rat fibroblasts were used to replace the human fibroblasts.

Preparation of CBSG graft for use on full-thickness wounds

Three different types of composite skin graft (CBSG) materials were compared and tested in animals, specifically laboratory rats. Type A CBSG consisted of allogenic rat fibroblasts seeded onto the basal side of a LASERSKIN™ artificial skin substratum. The fibroblasts were stimulated to produce collagen and other proteins by feeding the fibroblasts with DMEM supplemented with 10% FBS and 50µm ascorbic acid. After ten days, the LASERSKIN™ artificial skin substratum was turned over and the upper side was seeded with fibroblasts. The fibroblasts on both sides were then fed with DMEM supplemented with 10% FBS. When the fibroblasts on the upper side became sub-confluent, rat keratinocytes were seeded (3×10^4 cells/cm²) on top of the upper surface of CBSG material. The cells were fed with DMEM supplemented with 10% FBS, insulin (4mg/ml), cholera toxin (6ng/ml), EGF(10ng/ml). The keratinocytes became sub-confluent and were ready for grafting after only four to six days.

- J. Please amend the paragraph bridging pages 9 and 10 and the first three paragraphs of page 10 to recite:

Results

BIO
Twenty-four hours after the seeding on the CBSG substratum, the non-viable and unseeded keratinocytes were carefully washed away and concentrated for counting. There were no detectable 3T3 cells or fibroblasts detached from the LASERSKIN™ artificial skin during the washing procedure. The seeding efficacy of keratinocytes was calculated as:

$$\frac{[\text{Total keratinocytes count (before seeding)} - \text{non-seeded keratinocytes}]}{\text{Total keratinocytes count (before seeding)}} \times 100\%$$

SUB D7
It is evident that the selected type of CBSG substratum provided a suitable culture template for the in vitro proliferation of keratinocytes. On a plain LASERSKIN™ artificial skin, human keratinocytes showed a mean seeding efficacy of 75%. These human keratinocytes had a 95% seeding efficacy on LASERSKIN™ artificial skin populated with human fibroblasts and a 98% on 3T3 cell-seeded LASERSKIN™ artificial skin (Table 1). Rat keratinocytes had a seeding efficacy of 36% on plain LASERSKIN™ artificial skin. The respective seeding efficacies of rat keratinocytes on 3T3/ LASERSKIN™ artificial skin and on allogenic fibroblasts/ LASERSKIN™ artificial skin were 91% and 88%. The seeding efficacies of human/rat keratinocytes growing on 3T3 cell/ LASERSKIN™ artificial skin or on allogenic fibroblasts/ LASERSKIN™ artificial skin were significantly ($p < 0.001$) better than those seeded on plain LASERSKIN™ artificial skin, as noted in the Fisher Exact Testing using Stat Xact (version 2.05) statistical package.

It is believed that human/rat fibroblasts could achieve a role similar to that of the 3T3 cells in enhancing the seeding efficacies of keratinocytes growing on LASERSKIN™ artificial skin with respective p values of 0.445 and 0.646 using the same statistical package.

The optical transparency of the LASERSKIN™ artificial skin allowed regular inspection of the grafted wound bed as it healed. Skin biopsies were taken from the center of the grafted area of the subject. It was observed that the polypropylene ring prevented the migration of epithelium from the wound edge as no epithelial cell was found in the control rat wound sutured with polypropylene ring alone up to day 21. In sixteen out of the twenty (80%) animal wounds covered with Type A CBSG, the keratinocytes formed a multi-layered

SUB D7
CORR
B10
att

epithelium that had a basal layer in contact with underlying connective tissue. The undersurface of epidermis did not show rete. Fibrovascular ingrowth of connective tissue into the LASERSKIN™ artificial skin was observed. Eight (40%) of the Type B CBSG sites and seven (35%) of the Type C CBSG sites showed re-epithelization. Histologically there was no observable epithelium in 3 (15%), 10 (50%), and 12 (55%) of the grafted CBSG sites of Types A, B and C respectively (Table 2).

2

K. Please amend the paragraphs of the Discussion section from line 12, page 11 through line 10, page 12 to recite:

B11

Problems encountered in cultivation of keratinocytes on LASERSKIN™ artificial skin can be attributed to limited experience on this new product. Hyaluronic acid is a mucopolysaccharide with alternating β 1-3 glucuronidic and β 1-4 glycosaminidic bonds. LASERSKIN™ artificial skin mainly consists of 100% benzyl esterified hyaluronic acid. A weak electrostatic association exists between the keratinocytes and the LASERSKIN™ artificial skin. Since the volume and surface area of human keratinocytes differ from those of rat keratinocytes, there is a variation of charge density of two cell populations. Consequently they have different seeding efficacies on plain LASERSKIN™ artificial skin. The species-specific difference was abolished by cultivating keratinocytes on 3T3 cell/ LASERSKIN™ artificial skin because 3T3 cells were the dominant factors influencing keratinocyte attachment on LASERSKIN™ artificial skin.

The seeding efficacies of human and rat keratinocytes (3×10^4 cells/cm²) were 75% and 36% respectively on plain LASERSKIN™ artificial skin. The manufacturer of LASERSKIN™ artificial skin recommends seeding the human keratinocytes on the skin membrane pre-seeded with irradiated 3T3 cells. Good seeding efficiencies of 98% (human keratinocytes) and 91% (rat keratinocytes) were demonstrated on LASERSKIN™ for artificial skin with Mitomycin-treated 3T3 cells. In Green's method, keratinocytes are seeded on a 3T3 cell feeder layer which allows a rather low seeding density of $6-10 \times 10^4$ cells/cm² for the primary culture. A smaller seeding density of $2-5 \times 10^4$ cells/cm² is feasible for the secondary and tertiary cultures. The utility of cultured epidermal autograft grown on culture dish using Green's feeder layer technique is limited by the persistent 3T3 fibroblasts which sensitize the host to xenogenic antigen resulting in late graft rejection. The 3T3 cells were found less likely to be washed away from the LASERSKIN™ artificial skin material.

However, allogenic fibroblasts can achieve a similar role as 3T3 cells in initiation of keratinocyte colonization on LASERSKIN™ artificial skin.

B11
Cut

It is evident that the CBSG material consisting of cultured keratinocytes and dermal fibroblasts is a good human skin substitute for freshly excised full-thickness wounds which were previously grafted with allografts.

LASERSKIN™ Artificial Skin Preparation	Human Keratinocytes	Rat keratinocytes
Plain	75%	36%
with 3T3 cells	98%	91%
with human fibroblasts	95%	-
with rat fibroblasts	-	88%

Table 1 Mean Seeding Efficacies of Human and Rat Keratinocytes on LASERSKIN™ artificial skin. 15 determinations were made. Seeding efficacies of human/rat keratinocytes were significantly improved by 3T3 cells/fibroblasts ($p < 0.001$).

L. Please amend the paragraphs from line 4 on page 13 through line 10 on page 14 to recite:

B12

Attempts were made to graft freshly harvested epidermis onto INTEGRA™ neoderms in animal experiments. The dermal-epidermal separation was performed with dispase. The epidermal graft was not easily 'taken' by the neoderms whereas thin split thickness autografts readily incorporated on the neoderms as in many clinical applications. It is believed that the presence of fibroblasts in the dermis of the thin autograft may play an essential role in the keratinocyte attachment and its proliferation on the neoderms. It was observed that, during wound healing, cell-cell interactions between epidermal keratinocytes and dermal fibroblasts contributed to the organization of epidermis. Prostaglandin E2 (PGF2) is considered to be involved in the proliferation and differentiation of keratinocytes. The production of PEG2 was enhanced in the co-culture of keratinocytes and fibroblasts, whereas the PEG2 production was negligible in monolayer cultures of keratinocytes or fibroblasts. The fibroblasts also produced two soluble heparin-binding growth factors such as keratinocyte growth factor and hepatocyte growth factor/scatter factor that promote DNA synthesis and proliferation of keratinocytes.